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(54) Title: FLINT COMPOUNDS AND FORMULATIONS THEREOF

(57) Abstract: The present invention provides novel compounds, which comprise FLINT complexed with a divalent metal cation, pharmaceutical formulations thereof, methods for reducing and/or inducing aggregation of FLINT, and methods of using such compounds for treating or preventing diseases that may be related to the FasL/Fas interaction.

FLINT ANALOG COMPOUNDS AND FORMULATIONS THEREOF

Background of the Invention

The present invention is in the field of human medicine, particularly in the treatment and prevention of disorders that may be associated with the binding of FasL to the Fas receptor. More specifically, the present invention relates to compounds and formulations of a FLINT analog.

A number of tumor necrosis factor receptor proteins ("TNFR proteins") and proteins homologous thereto have been isolated in recent years. They have many potent biological effects and aberrant activity of these proteins has been implicated in a number of disease states.

One such TNFR homologue, reported in July, 1998 (Gentz *et al.*, WO 98/30694), binds the protein FAS Ligand and thereby inhibits the activation of another TNFR homologue, FAS, by FAS Ligand (U.S. Provisional Applications Serial Nos. 60/112,577, 60/112,933, and 60/113,407, filed December 17, 18 and 22, 1998, respectively). This new protein is referred to herein as "FAS Ligand Inhibitory Protein" or "FLINT."

Over activation of FAS by FAS Ligand has been implicated in a number of pathological conditions, including runaway apoptosis (Kondo *et al.*, Nature Medicine 3(4):409-413 (1997) and Galle *et al., J. Exp. Med.* 182:1223-1230 (1995)) and inflammatory disease resulting from neutrophil activation (Miwa *et al.,. Nature Medicine 4:*1287 (1998)).

"Runaway apoptosis" is a level of apoptosis greater than normal or apoptosis occurring at an inappropriate time. Pathological conditions caused by runaway apoptosis include organ failure, for example in the liver, kidneys and pancreas. Inflammatory diseases

associated with excessive neutrophil activation include, but are not limited to, sepsis, ARDS, SIRS and MODS.

The structural properties of proteins may be affected by divalent cations. For example, aggregation and/or precipitation of proteins, as well as oligomerization, may be induced by divalent cations. Aggregation of proteins can impact the ability to produce, purify, formulate and deliver a protein, for example, as a pharmaceutical product. Moreover, aggregation and/or oligomerization can impact the stability of a protein, for example, in storage. In some instances, a protein's stability can be enhanced if aggregated and/or precipitated prior to, or during storage.

FLINT and analogs thereof, for example, analog R218Q, aggregate and eventually precipitate from solution when exposed to divalent cation. For example, analog R218Q purified by IMAC chromatography and elution in 0.4M imidazole, precipitates from solution (See Example 7, *infra*). These observations suggest that FLINT and analogs thereof interact with divalent cations, such as Ni^{+2}, to cause aggregation and/or precipitation.

As FLINT analogs are potentially useful therapeutic proteins, their purification and formulation are important factors to be worked out on the path to development of a pharmaceutical product. While FLINT is known from prior disclosures (See e.g WO 98/30694 and WO 99/50413), its formulation has not been thoroughly investigated, nor has the impact of divalent cation on the aggregation and/or oligomerization of the protein and its analogs been sufficiently investigated for purposes of realizing the full therapeutic and pharmaceutical utility.

The present invention relates to a method for eliminating aggregation and/or precipitation of FLINT analog(s), useful in purifying one or more FLINT analogs comprising the removal of divalent cation from a solution or other medium comprising FLINT analog(s).

The invention relates further to the purification of FLINT analogs from a solution of one or more of said FLINT analogs, by immobilized metal ion affinity (IMAC) chromatography, comprising removal of divalent cation from said solution.

The invention relates further to a composition comprising a FLINT analog and a divalent metal cation.

The invention relates further to a method for producing a composition comprising FLINT analog, in association with a divalent cation.

The present invention relates further to a pharmacuetical formulation comprising FLINT analog, in association with a divalent metal cation, and with one or more pharmaceutically acceptable carriers, diluents, or excipients.

Accordingly, the present invention provides a FLINT analog-divalent cation complex, which comprises a FLINT analog complexed with a divalent metal cation, pharmaceutical formulations thereof, and methods for using such pharmaceutical formulations thereof, and methods of using such compounds for the treatment and/or prevention of disorders that may be associated with the binding of Fas to FasL, and/or LIGHT to the LTβR and/or TR2/HVEM receptors.

Compounds such as FLINT which inhibit the binding of FAS to FAS Ligand or LIGHT to LTβR and/or TR2/HVEM receptors can be used to treat or prevent diseases or conditions associated with these binding interactions.

Exemplary FLINT analogs of the invention suitable for formulation with a divalent cation have an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing tryptophan at position 53 with aspartic acid;

b) replacing threonine at position 88 with proline;

c) replacing alanine at position 107 with serine, aspartic acid, glutamic acid or threonine;

d) replacing isoleucine at position 110 with threonine or glutamic acid; or

e) replacing proline at position 104 with serine.

In another aspect, a FLINT analog has an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing alanine at position 2 or position 12 with asparagine;

b) replacing proline at position 25, position 38, position 126 or position 171 with asparagine;

c) replacing arginine at position 35 with asparagine;

d) replacing serine at position 37 with asparagine and proline at position 38 with any other naturally occurring amino acid;

e) replacing serine at position 166 with asparagine;

f) replacing leucine at position 172 with asparagine;

g) replacing aspartic acid at position 194 with asparagine;

h) replacing glycine at position 114 with asparagine and proline at position 115 with any naturally occurring amino acid; or

i) replacing arginine at position 218 with asparagine.

In yet another aspect, a FLINT analog has an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing asparagine at position 63 with tryptophan;

b) replacing glycine at position 67 with aspartic acid and replacing alanine at position 94 or glycine at position 95 with tyrosine;

c) replacing arginine at position 69 with glutamic acid;

d) replacing arginine at position 82 with glutamic acid or threonine;

e) replacing alanine at position 94 with tyrosine and replacing glycine at position 95 with aspartic acid;

f) replacing phenylalanine at position 96 with glutamine;

g) replacing alanine at position 101 with threonine; or

h) replacing glycine at position 95 with aspartic acid.

In yet another aspect, a FLINT analog has an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing arginine at position 10 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then alanine at position 12 is optionally replaced with serine or threonine;

b) replacing glutamic acid at position 13 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then glycine at position 15 is optionally replaced with serine or threonine;

c) replacing glutamic acid at position 16 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then leucine at position 18 is optionally replaced with serine or threonine;

d) replacing arginine at position 17 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then

valine at position 19 is optionally replaced with serine or threonine;

e) replacing arginine at position 31 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then cysteine at position 33 is optionally replaced with serine or threonine;

f) replacing arginine at position 34 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then aspartic acid at position 36 is optionally replaced with serine or threonine;

g) replacing arginine at position 35 with glutamine, asparagine, serine or threonine;

h) replacing aspartic acid at position 36 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then proline at position 38 is optionally replaced with serine or threonine;

i) replacing arginine at position 143 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is asparagine, then cysteine at position 145 is optionally replaced with serine or threonine; or

j) replacing aspartic acid at position 161 with glutamine, asparagine, serine or threonine, provided that when the replacing amino acid is aspargine, then leucine at position 163 is optionally replaced with serine or threonine.

Optional replacement by serine or threonine of an amino acid two positions removed from the replacing asparagine in the direction of the *C*-terminus creates new *N*-linked glycosylation site motifs, NXS/T. FLINT analogs with new glycosylation site motifs are preferably prepared from recombinant mammalian host cells that express a gene encoding said polypeptide, thereby preparing an *N*-

glycosylated product. Glycosylation site motifs are discussed in greater detail hereinbelow.

In yet another embodiment, the present invention relates to a FLINT analog-divalent cation complex comprising SEQ ID NO:1 having the amino acid sequence modified by:

a) replacing alanine at position 2, 12, 107, 179 or 209 with threonine;

b) replacing threonine at position 4 or 162 with alanine;

c) replacing valine at position 1 or isoleucine at position 110 with methionine;

d) replacing glutamic acid at position 13 with aspartic acid;

e) replacing arganine at position 17 with tryptophan;

f) replacing alanine at position 75 with proline;

g) replacing serine at positione 102 with leucine;

h) replacing glycine at position 169 with alanine;

i) replacing glutamic acid at position 183 with lysine;

j) replacing glutamine at position 225 with arginine;

k) replacing glycine at position 237 with glutamic acid; or

l) replacing valine at position 270 with glycine,

said fragment comprising amino acids 49-165 of the polypeptide; and

physiologically acceptable salts thereof.

In another aspect, a FLINT analog-divalent complex comprises an amino acid sequence of SEQ ID NO:1 modified by:

a) replacing alanine at position 2 or alanine at position 12 with asparagine;

b) replacing proline at position 25, or proline at position 38, or proline at position 126, or proline at position 171 with asparagine;

c) replacing arginine at position 35 with asparagine;

d) replacing serine at position 37 with asparagine, and proline at position 38 with any naturally occurring amino acid;

e) replacing serine at position 166 with asparagine;

f) replacing leucine at position 172 with asparagine;

g) replacing aspartic acid at position 194 with asparagine;

h) replacing threonine at position 114 with asparagine and proline at position 115 to any naturally occurring amino acid; or

i) replacing arginine at position 218 with asparagine.

In yet another aspect, a FLINT analog-divalent cation complex has an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing alanine at position 12 with asparagine and optionally replacing glutamic acid at position 13 with glutamine;

b) replacing arginine at position 34 with asparagine and replacing aspartic acid at position 36 with threonine;

c) replacing arginine at position 35 with asparagine and optionally replacing serine at position 37 with threonine;

d) replacing serine at position 132 with asparagine and optionally replacing serine at position 134 with threonine;

e) replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

f) replacing arginine at position 35 and aspartic acid at position 194 with asparagine;

g) replacing alanine at position 12 with asparagine, optionally replacing glutamic acid at position 13 with glutamine, replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

h) replacing arginine at position 34 with asparagine, replacing aspartic acid at position 36 with threonine, replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

i) replacing arginine at position 35 and aspartic acid at position 194 with asparagine and replacing serine at position 37 and/or position 196 with threonine; or

j) replacing arginine at position 218 with glutamine.

k) replacing glycine at position 26 with aspartic acid and replacing serine at position 132 with asparagine;

l) replacing alanine at position 12 with asparagine, replacing serine at position 132 with asparagine, and replacing serine at position 134 with threonine; or

m) replacing threonine at position 216 with proline and replacing arginine at position 218 with glutamine.

The present invention provides conditions under which potency and/or stability of FLINT analogs may be significantly enhanced. Thus, effective pharmacological treatment may be achieved at lower doses thereby abrogating toxic or other undesirable side effects. Accordingly, the present invention provides a protein-cation complex, which comprises a FLINT analog, or FLINT fusion protein comprising a FLINT analog complexed with a divalent metal cation.

Summary of the Invention

The invention provides a composition comprising a FLINT analog or fusion protein comprising a FLINT analog complexed with a divalent metal cation. The invention additionally provides parenteral pharmaceutical formulations comprising a FLINT analog divalent cation composition and methods for using such compounds for treating or preventing diseases and disorders that may be associated with the binding of Fas to FasL, and/or LIGHT to LTβR and/or TR2/HVEM receptors. The invention further provides a process of preparing such compounds, which comprises combining a FLINT analog or fusion protein comprising a FLINT analog and a divalent metal cation in an aqueous solution at a pH of about 4.5 to 9.0.

Detailed Description and Preferred Embodiments

For purposes of the present invention, as disclosed and claimed herein, the following terms and abbreviations are defined as follows:

The term "aggregate" or "aggregation" refers to a non-covalent association of protein or peptide molecules

including monomers, subunits, and fragments thereof, that may lead to precipitation of said molecules.

"FLINT protein analog," "FLINT analog," or "analog" refers to a protein derivative of mature FLINT (SEQ ID NO:1) or native FLINT (SEQ ID NO:2) comprising one or more amino acid deletions, additions, substitutions, inversions, or changes in the glycosylation pattern of residues within SEQ ID NO:1 or SEQ ID NO:2. Also included in the term are FLINT fusion proteins

"FLINT" is used herein to encompass FLINT analogs and FLINT fusion proteins.

"FLINT glycosylation mutant" or "glycosylation mutant" as used herein refers to amino acid changes in FLINT by which asparagine is substituted for the wild type residue to create surface accessible glycosylation sites. Computer and homology models predict that these amino acid residues are on the surface of FLINT and are distinct from the site on FLINT that binds FAS Ligand and therefore contribute minimally to FLINT/FAS Ligand binding affinity. As a consequence, it is expected that FLINT analogs obtained by replacing these surface accessible amino acid residues with asparagine would be glycosylated at the new glycosylation sites, would retain their affinity for FAS Ligand and will exhibit improved pharmaceutical and pharmacological properties. Specifically, glycosylation mutants comprise modified FLINT polypeptides having an amino acid sequence of SEQ ID NO: 1, modified by:

a) replacing alanine at position 12 with asparagine and optionally replacing glutamic acid at position 13 with glutamine;

b) replacing arginine at position 34 with asparagine and replacing aspartic acid at position 36 with threonine;

c) replacing arginine at position 35 with asparagine and optionally replacing serine at position 37 with threonine;

d) replacing serine at position 132 with asparagine and optionally replacing serine at position 134 with threonine;

e) replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

f) replacing arginine at position 35 and aspartic acid at position 194 with asparagine;

g) replacing alanine at position 12 with asparagine, optionally replacing glutamic acid at position 13 with glutamine, replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

h) replacing arginine at position 34 with asparagine, replacing aspartic acid at position 36 with threonine, replacing aspartic acid at position 194 with asparagine and optionally replacing serine at position 196 with threonine;

i) replacing arginine at position 35 and aspartic acid at position 194 with asparagine and replacing serine at position 37 and/or position 196 with threonine;

j) replacing glycine at position 26 with aspartic acid and replacing serine at position 132 with asparagine;

k) replacing alanine at position 12 with asparagine, replacing serine at position 132 with asparagine, and replacing serine at position 134 with threonine.

The term "fusion protein" or "FLINT fusion protein" as used herein refers to a FLINT protein or analog thereof wherein said protein or analog is fused to a heterologous protein or peptide including a peptide tag useful in purification, e.g. a His-tag.

The term "negatively charged group" or "negatively charged amino acid" refers to Asp or Glu.

The term "positively charge group" or "positively charged amino acid" refers to His, Arg, or Lys.

The term "polar uncharged" or "polar uncharged amino acid" refers to Cys, Thr, Ser, Gly, Asn, Gln, and Tyr.

The term "nonpolar" or "nonpolar amino acid" refers to Ala, Pro, Met, Leu, Ile, Val, Phe, or Trp.

The term "naturally-occurring amino acid" refers to any of the 20 L-amino acids that are found in proteins.

"Treating" as used herein, describes the management and care of a patient for the purpose of combating the disease, condition, or disorder and includes the administration of a protein of the present invention to prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

"Isotonicity agent" refers to an agent that is physiologically tolerated and embarks a suitable tonicity to the formulation to prevent the net flow of water across the cell membrane. Compounds, such as glycerin, are commonly used for such purposes at known concentrations. Other possible isotonicity agents include salts, e.g., NaCl, dextrose, and lactose.

The term "oligomer" or "oligomerization" refers to a specific interaction of more than one protein subunit in non-covalent or covalent fashion. Examples of specific oligomers would include dimers, trimers, tetramers, etc. As used herein the term refers to association of one or more FLINT analogs including association of identical or non-identical subunits such as, for example, non-identical FLINT analogs in association. The process of oligomerization lies on a continuum with the process of aggregation, the latter representing non-specific interactions, that in the extreme, lead to precipitation.

"Physiologically tolerated buffer" refers to buffers including TRIS, sodium acetate, sodium phosphate, or sodium citrate. The selection and concentration of buffer is known in the art.

"Pharmaceutically acceptable preservative" refers to a multi-use parenteral formulation that meets guidelines for preservative effectiveness to be a commercially viable product. Pharmaceutically acceptable preservatives known in the art as being acceptable in parenteral formulations include: phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, p-cresol, phenylmercuric nitrate, thimerosal and various mixtures thereof. Other preservatives may be found, e.g., in Wallhauser, K. H., Develop. Biol. Standard 24, 9-28 (Basel, S. Krager, 1974). The concentration necessary to achieve preservative effectiveness is dependent upon the preservative used and the conditions of the formulation.

As noted above, the invention provides a compound comprising a FLINT analog complexed with a divalent metal cation.

Applicants have discovered that FLINT analogs undergo oligomeriztion and/or aggregation in the presence of divalent cations. In one aspect of the present invention, pharmaceutical compositions of FLINT analog and divalent cation provide depot formulations for therapeutic use. In another aspect, oligomerization and/or aggregation of FLINT can be reduced, prevented, or reversed by removal of divalent cation from said protein. In this aspect, the invention relates to a process or method for purifying FLINT and for maintaining FLINT in solution.

The presently claimed compounds comprise FLINT analogs complexed with a divalent metal cation. A divalent metal cation includes, for example, Zn^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, Cd^{+2}, Ca^{+2}, Ni^{+2} and the like. A combination of two or more divalent metal cations is operable; however the preferred compounds comprise a single species of metal cation, most preferably Zn^{++}. Preferably, the divalent metal cation is in excess; however, the molar ratio of at least one molecule of a divalent metal cation for each ten molecules of FLINT analog is operable. Preferably, the compounds comprise from 1 to 100 divalent metal cations per molecule of FLINT analog. The compounds may be amorphous or crystalline solids.

Appropriate forms of metal cations are any form of a divalent metal cation that is available to form a complex with a molecule of FLINT analog of the present invention. The metal cation may be added in solid form or it may be added as a solution. Several different cationic salts can be used in the present invention. Representative examples of metal salts include the acetate, bromide, chloride, fluoride, iodide and sulfate salt forms. The skilled artisan will recognize that there are many other

metal salts which also might be used in the production of the compounds of the present invention. Preferably, zinc acetate or zinc chloride is used to create the zinc-FLINT analog compounds of the present invention. Most preferably, the divalent metal cationic salt is zinc chloride.

Generally, the claimed compounds are prepared by techniques known in the art. For example, convenient preparation is to combine FLINT analog with the desired divalent metal cation in an aqueous solution at a pH of about 4.5-9.0, preferably about pH 5.5-8, most preferably, pH 6.5-7.6. The claimed compound precipitates from the solution as a crystalline or amorphous solid. Significantly, the compound is easily isolated and purified by conventional separation techniques appreciated in the art including filtration and centrifugation. Significantly, the protein-metal cation complex is stable and may be conveniently stored as a solid or as an aqueous suspension.

The present invention further provides a pharmaceutical formulation comprising a compound of the present invention and water. The concentration of the FLINT analog in the formulation is about 0.1 mg/mL to about 100 mg/mL; preferably about 0.5 mg/mL to about 50.0 mg/mL; most preferably, about 5.0 mg/mL.

The formulation preferably comprises a pharmaceutically acceptable preservative at a concentration necessary to maintain preservative effectiveness. The relative amounts of preservative necessary to maintain preservative effectiveness varies with the preservative used. Generally, the amount necessary can be found in

Wallhauser, K. H., Develop. Biol. Standard 24, 9-28 (Basel, S. Krager, 1974), herein incorporated by reference.

An isotonicity agent, preferably glycerin, may be added to the formulation. The concentration of the isotonicity agent is in the range known in the art for parenteral formulations, preferably about 16 mg/mL glycerin. The pH of the formulation may also be buffered with a physiologically tolerated buffer. Acceptable physiologically tolerated buffers include TRIS, sodium acetate, sodium phosphate, or sodium citrate. The selection and concentration of buffer is known in the art.

Other additives, such as a pharmaceutically acceptable excipients like Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 80 (polyoxyethylene (20) sorbitan monooleate), Pluronic F68 (polyoxyethylene polyoxypropylene block copolymers), BRIJ 35 (polyoxyethylene (23) lauryl ether), and PEG (polyethylene glycol) may optionally be added to the formulation to reduce aggregation.

The claimed pharmaceutical formulations are prepared in a manner known in the art, and are administered individually or in combination with other therapeutic agents. The formulations of the present invention can be prepared using conventional dissolution and mixing procedures. Preferably, the claimed formulations are prepared in an aqueous solution suitable for parenteral use. That is, a protein solution is prepared by mixing water for injection, buffer, and a preservative. Divalent metal cations are added to a total cation concentration of about 0.001 to 5.0 mg/mL, preferably 0.05 to 1.5 mg/mL. The pH of the solution may be adjusted to completely

precipitate the FLINT analog-cation complex. The compound is easily resuspended before administration to the patient.

Parenteral daily doses of the compound are in the range from about 1 ng to about 10 mg per kg of body weight, although lower or higher dosages may be administered. The required dosage will be determined by the physician and will depend on the severity of the condition of the patient and upon such criteria as the patient's height, weight, sex, age, and medical history.

Variations of this process would be recognized by one of ordinary skill in the art. For example, the order the components are added, if a the surfactant is used, the temperature, and pH at which the formulation is prepared may be optimized for the concentration and means of administration used.

The pH of the formulation is generally pH 4.5 to 9.0 and preferably 5.5 to 8.0, most preferably 6.5 to 7.6; although more acidic pH wherein a portion or all of the protein-metal cation complex is in solution is operable.

The formulations prepared in accordance with the present invention may be used in a syringe, injector, pumps or any other device recognized in the art for parenteral administration.

The proteins used in the present compounds can be prepared by any of a variety of recognized peptide synthesis techniques including classical (solution) methods, solid phase methods, semi synthetic methods, and more recent recombinant DNA methods. Recombinant methods are preferred if a high yield is desired. The basic steps in the recombinant production of protein include:

a) construction of a synthetic or semi-synthetic (or isolation from natural sources) DNA encoding the FLINT analog,

b) integrating the coding sequence into an expression vector in a manner suitable for the expression of the protein either alone or as a fusion protein,

c) transforming an appropriate eukaryotic or prokaryotic host cell with the expression vector, and

d) recovering and purifying the recombinantly produced protein.

A cDNA encoding native FLINT (SEQ ID NO:3) can provide a template from which to engineer specific mutations that result in a nucleic acid that encodes an analog of the invention. For example, FLINT cDNA is used as a template for introducing appropriate point mutations (i.e. construction of FLINT analog cDNAs). A suitable protocol is described in detail in "Current Protocols in Molecular Biology", volume 1, section 8.5.7 (John Wiley and Sons, Inc. publishers), incorporated herein by reference. Briefly, synthetic oligonucleotides are designed to incorporate one or more desired point mutation(s) at one end of an amplified fragment, e.g. at position 218 of SEQ ID NO:1. Following first strand PCR, the amplified fragments encompassing the mutation are annealed with each other and extended by mutually primed synthesis. Annealing is followed by a second PCR step utilizing 5′ forward and 3′ reverse end primers in which the entire mutagenized fragment gets amplified and is ready for subcloning into the appropriate vector.

Synthetic genes and nucleic acids can be constructed by techniques well known in the art. Owing to the degeneracy of the genetic code, the skilled artisan will recognize that multiple DNA sequences may be constructed which encode the desired proteins. Synthesis is achieved by recombinant DNA technology or by chemical synthesis, for example, see Brown, et al. (1979) Methods in Enzymology, Academic Press, N.Y., Vol. 68, pgs. 109-151. A DNA sequence(s) encoding FLINT analogs can be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). It may be desirable in some applications to modify the coding sequence of a FLINT analog so as to incorporate a convenient protease sensitive cleavage site, e.g., between the signal peptide and the structural protein facilitating the controlled excision of the signal peptide from the fusion protein construct.

A gene encoding FLINT analog(s) may also be created by using the polymerase chain reaction (PCR). The template can be a cDNA library, for example (commercially available from CLONETECH or STRATAGENE). Such methods are well known in the art, *c.f.* Maniatis, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), herein incorporated by reference.

The constructed or isolated DNA sequences are useful for expressing FLINT analog. When the sequences comprise a fusion gene, the resulting product, if desired, can be treated enzymatically or chemically to release FLINT. A variety of peptidases which cleave a polypeptide

at specific sites or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase) of the peptide chain are known. Furthermore, particular chemicals (e.g. cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. See U.S. Patent No. 5,126,249; Carter P., Site Specific Proteolysis of Fusion Proteins, Ch. 13 in Protein Purification: From Molecular Mechanisms to Large Scale Processes, American Chemical Soc., Washington, D.C. (1990).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with these hosts. The vector ordinarily carries a replication origin and one or more sequences for selection of transformed cells.

The desired coding sequence is inserted into an expression vector in the proper orientation to be transcribed from a promoter and ribosome binding site, both of which should be functional in the host cell in which the protein is to be expressed.

In general, procaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains

which may be used include E. coli B and E. coli X1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

The DNA molecules may also be recombinantly expressed in eukaryotic expression systems. Preferred promoters controlling transcription in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. •-actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers, et al., Nature, 273:113 (1978). The entire SV40 genome may be obtained from plasmid pBRSV, ATCC 45019. The immediate early promoter of the human cytomegalovirus may be obtained from plasmid pCMBb (ATCC 77177). Of course, promoters from the host cell or related species also are useful herein.

Transcription of the DNA by higher eucaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively oriented and positioned independently and have been found 5' (Laimins, L. et al., PNAS 78:993 (1981)) and 3' (Lusky, M. L., et al., Mol. Cell Bio. 3:1108 (1983)) to the transcription unit, within an intron (Banerji, J. L. et al., Cell 33:729 (1983)) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell Bio. 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, alpha-

fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding protein. The 3' untranslated regions also include transcription termination sites.

Expression vectors may contain a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR, which may be derived from the BglII/HindIII restriction fragment of pJOD-10 [ATCC 68815]), thymidine kinase (herpes simplex virus thymidine kinase is contained on the BamHI fragment of vP-5 clone [ATCC 2028]) or neomycin (G418) resistance genes (obtainable from pNN414 yeast artificial chromosome vector [ATCC 37682]). When such selectable markers are successfully transferred into a mammalian host cell, the transfected mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow without a supplemented media. Two examples are: CHO DHFR$^-$ cells (ATCC CRL-9096) and mouse LTK$^-$ cells (L-M(TK-) ATCC CCL-2.3). These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these

cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in nonsupplemented media.

A suitable vector for eucaryotic expression is pRc/CMV. pRc/CMV is commercially available from Invitrogen Corporation, 3985 Sorrento Valley Blvd., San Diego, CA 92121. To confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain DH10B (ATCC 31446) and successful transformants selected by antibiotic resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequence by the method of Messing, et al., Nucleic Acids Res. 9:309 (1981).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring

Harbor, New York (1989), or Current Protocols in Molecular Biology (1989) and supplements.

Suitable host cells for expressing the vectors encoding the claimed proteins in higher eucaryotes include: African green monkey kidney line cell line transformed by SV40 (COS-7, ATCC CRL-1651); transformed human primary embryonal kidney cell line 293,(Graham, F. L. et al., J. Gen Virol. 36:59-72 (1977), Virology 77:319-329, Virology 86:10-21); baby hamster kidney cells (BHK-21(C-13), ATCC CCL-10, Virology 16:147 (1962)); Chinese hamster ovary cells CHO-DHFR$^-$ (ATCC CRL-9096), mouse Sertoli cells (TM4, ATCC CRL-1715, Biol. Reprod. 23:243-250 (1980)); African green monkey kidney cells (VERO 76, ATCC CRL-1587); human cervical epitheloid carcinoma cells (HeLa, ATCC CCL-2); canine kidney cells (MDCK, ATCC CCL-34); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); human diploid lung cells (WI-38, ATCC CCL-75); human hepatocellular carcinoma cells (Hep G2, ATCC HB-8065);and mouse mammary tumor cells (MMT 060562, ATCC CCL51).

In addition, unicellular eukaryotes such as yeast may also be used. Saccharomyces cerevisiae, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb, et al., Nature 282:39 (1979); Kingsman et al., Gene 7:141 (1979); Tschemper et al., Gene 10:157 (1980)) is commonly used. This plasmid already contains the trp gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85:12 (1977)).

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD ATCC 53231 and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 ATCC 39532), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 ATCC 57090, 57091), zymomonas mobilis (United States Patent No. 5,000,000 issued March 19, 1991), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which contain inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV ATCC 39475, United States Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose (GAL1 found on plasmid pRY121 ATCC 37658) utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman _et al_., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal from _Saccharomyces cerevisiae_ (found in conjunction with the CYC1 promoter on plasmid YEpsec--hI1beta ATCC 67024), also are advantageously used with yeast promoters.

The following examples are provided to further illustrate the preparation of the formuations of the invention. The scope of the invention is not construed as merely consisting of the following examples.

EXAMPLE 1

Preparation of FLINT analog-Zinc Formulations

About 20 mg of a FLINT analog in which the arginine residue at position 34 of SEQ ID NO:1 is replaced by asparagine, the aspartic acid at position 36 is replaced by threonine, the aspartic acid at position 194 is replaced by asparagine, and the serine at position 196 is replaced by threonine (hereinafter referred to as "RDDS") is completely dissolved in 32 mL of an aqueous solution containing 16 mg/mL glycerin and 2 mg/mL phenol and passed through a sterile 0.2 μ filter. An aqueous solution containing 100 mg/mL of zinc in water is prepared from zinc chloride. Dilutions are made to prepare 10 mg/mL zinc and 1 mg/mL zinc solutions. Five 6-mL aliquots of the RDDS solution are mixed with the zinc solutions as shown in Table I:

Table I

Sample	mL of 1 mg/mL zinc added	ML of 10 mg/mL zinc added	ML of 100 mg/mL zinc added	ml of H_2O added	Total mg/mL zinc concentration
A	0	0	0	100	0
B	17	0	0	83	0.0027
C	0	33	0	67	0.054
D	0	0	19	81	0.30
E	0	0	92	8	1.50

Each formulation is adjusted to pH 7.48 ± 0.03 using small volumes of 2N and 5N sodium hydroxide and stored at 4°C. Sample A is completely clear while samples B through E are cloudy suspensions.

EXAMPLE 2

Analysis of Zinc Formulations

Size-exclusion chromatography is performed on the centrifuged supernatants of Samples A through E of Example 1. For these analyses, 100 uL of the supernatants are injected onto an analytical Superdex-75® (3.2/30, Pharmacia) column equilibrated in PBS (Dulbecco's Phosphate-Buffered Saline, GibcoBRL). The column is eluted at ambient temperature at 0.5 mL/min and the protein in the eluant monitored at 214 nm.

EXAMPLE 3

Biological Activity of the Zinc Formulations

A FLINT analog bioassay measuring cell survival (i.e. prevention of apoptosis) is performed in a 96 well plate format with reactions of 100 μl/well. 25 μl of Jurkat cells ($5X10^4$ cells/well) is mixed with 25 μl of recombinant human FasL (final concentration 150ng/ml) and 50 μl of FLINT analog in Example 1. Cells are incubated at 37^0C overnight. Twenty μl of MTS tetrazolium compound (U.S. Pat. No. 5,185,450 assigned to the Univ. of South Florida and exclusively licensed to Promega Corporation, Madison, WI) is added to each well and the incubation carried out for 2h at 37^CC. Absorbance at 490 nm is recorded using a plate reader.

EXAMPLE 4

Large Scale FLINT Analog Polypeptide Purification

Large scale production of a FLINT analog, RDDS (containing a 6 histidine tag) is performed by growing stable pools in several roller bottles. After reaching confluency, cells are further incubated in serum-free medium

for 5 to 7 days to secrete maximum amount of FLINT analog into the medium. Media containing FLINT analog is adjusted to 0.1 % CHAPS concentrated in an Amicon ProFlux M12 tangential filtration system to 350 ml using an Amicon S3Y10 UF membrane. The concentrated media is passed over IMAC (Immobilized Metal-Affinity Chromatography (Pharmacia, 5 to 20 ml column) at a flow rate of 1 ml/min. The column is washed with buffer A (PBS (1 mM potassium phosphate, 3 mM sodium phosphate), 0.5 M NaCl, pH 7.4) until the absorbence returns to baseline and the bound polypeptides is eluted with a linear gradient from 0.025 M to 0.5 M Imidazol (in buffer A) developed over 60 min. Fractions containing FLINT analog are pooled and EDTA is added to a final concentration of 50 mM EDTA. The pooled fractions containing FLINT analog are concentrated using an Ultrafree centrifugal filter unit (Millipore, 10 kDa molecular weight cut-off) to 2 ml. This material is passed over a Superdex 75 (Pharmacia, 16/60) sizing column equilibrated with PBS, 0.5 M NaCl, pH 7.4, at a flow rate of 1 ml/min. Fractions containing FLINT analog are analyzed by SDS-PAGE. The N-terminal sequence of FLINT analog is confirmed on the purified polypeptide.

EXAMPLE 5

Interaction of R34N D36T His-tagged FLINT Analog With Ni^{2+}

FLINT analog R34N, D36T His-tagged (50 ul) is incubated with $NiCl_2$ (final concentration of 1 mM) or with $NiCl_2$ and EDTA (both at a final concentration of 1 mM) for at 4 °C for 2 hours. As a control, R34N, D36T His-tagged FLINT is treated without the addition of $NiCl_2$ or EDTA. After the incubation, the samples are centrifuged in an Eppendorf centrifuge at maximum speed for 5 min. 20 µl of each sample is injected on to an analytical Superdex 75 column and eluted from this column at a flow rate of 70 µl/min in PBS, 0.5 M NaCl, pH 7.4.

EXAMPLE 6

Effect of Divalent Cation on FLINT and Analogs

FLINT and FLINT analogs were purified from either AV12 or 293 cell lines. Protein samples were stored in PBS at pH 7.4, 0.5 M NaCl, and 10% glycerol. The effect of divalent cations, such as Ni^{2+}, Zn^{2+} and Ca^{2+}, was investigated using intrinsic tryptophan fluorescence intensity and fluorescence anisotropy. Since fluorescence anisotropy is very sensitive to the rotational correlation time of the molecule, the change in the value of anisotropy reflects change in the association of FLINT molecules upon addition of divalent cations.

Concentrations of FLINT or FLINT analogs were measured on an AVIV model 14DS spectrometer. Spectra were collected from 400 nm to 260 nm at 1-nm bandwidth and were corrected for the solvent and scatter using data obtained between 360 nm to 320 nm by the AVIV computer program Loggen. The peak absorbance at about 280 nm was divided by 0.786 $mg^{-1}\ cm^{-1}$ to determine the concentration of the protein in a 1-cm pathlength cell. 5 mM $NiCl_2$ or $ZnCl_2$ or $CaCl_2$ stock solution was made by dissolving in H_2O the appropriate amount of solid $NiCl_2$, $ZnCl2_2$, or $CaCl_2$.

Tryptophan fluorescence intensity and fluorescence anisotropy were measured using an ISS PCI photon counting spectrofluorometer. A protein solution of about 0.1 mg/ml concentration was excited at 295 nm and the total intensity of fluorescence and fluorescence anisotropy was recorded using a 335 nm cutoff filter in a cell of 5 mm x 10 mm pathlength with a 8 nm excitation bandwidth. A small aliquot of 5 mM $NiCl_2$, $ZnCl_2$ or $CaCl_2$ stock was added to the protein sample in the cell to adjust the

concentration of divalent cation concentration. The sample was then mixed by inverting the cuvette after each addition of divalent metal . The fluorescence signal intensity and anisotropy were determined as a function of divalent ion concentration.

The fluorescence intensity and anisotropy data obtained on FLINT as a function of $NiCl_2$ or $ZnCl_2$ is shown in Table I. Addition of either $NiCl_2$ or $ZnCl_2$ decreased the fluorescence intensity and increased the anisotropy, indicating an association of FLINT molecules. The association of FLINT molecules upon addition of $ZnCl_2$ is reversible by addition of 2 mM EDTA, as indicated by the decrease of anisotropy to the initial anisotropy value in the absence of $ZnCl_2$.

Table I. Typtophan fluorescence intensity and anisotropy of FLINT as a function of $NiCl_2$ or $ZnCl_2$ concentration.

[NiCl2] uM	Intensity	Anisotropy	[ZnCl2] uM	Anisotropy
0	669978	0.1357	0	0.1344
5	648081	0.14	5	0.1304
10	628453	0.1368	10	0.1273
20	578457	0.1396	20	0.1342
40	548057	0.1416	40	0.1462
80	514699	0.1461	80	0.1724
100	457960	0.1527	100	0.1884
200	443962	0.1578	200	0.2172
400	361070	0.1671	400	0.2436
+ 2 mM EDTA	442029	0.1528	2 mM EDTA	0.1338

The effect of $NiCl_2$ and $ZnCl_2$ on His-tagged R218Q FLINT was also investigated. In contrast to FLINT, addition of small concentration of $NiCl_2$ or $ZnCl_2$ causes

precipitation of His-tagged R218Q, leading to the rapid increase of fluorescence anisotropy, as shown in Table II. The precipitation caused by $ZnCl_2$ can be readily reversed by addition of 2 mM EDTA. However, the precipitation by $NiCl_2$ can only be reversed very slowly.

Table II. Fluorescence anisotropy of His-tagged R218Q FLINT as a function of $NiCl_2$ or $ZnCl_2$ concentration.

[NiCl2] uM	Anisotropy	[ZnCl2] uM	Anisotropy
0	0.1351	0	0.1299
5	0.1985	5	0.1360
10	0.214	10	0.2293
		+ 2 mM EDTA	0.1306

His-tagged analog RDDSR (i.e. R34N/D36T/D194N/S196T/R218Q) FLINT was purified from transiently-transfected 293EBNA cell line. This analog contains two additional putative asparagine-linked glycosylation sites at Asn34 and Asn194. Fluorescence intensity and anisotropy as a function of divalent cation concentration are shown in Table III. In comparison to His-tagged R218Q, the hyperglycosylated His-tagged RDDSR is much less sensitive to $NiCl_2$. Addition of $NiCl_2$ up to 400 uM did not cause visible precipitation of protein. However, $ZnCl_2$ does cause the protein to precipitate, although to a lesser degree compared to His-tagged R218Q FLINT. The precipitated sample dissolved rapidly with addition of 1 mM EDTA and the anisotropy returned to the initial value in the absence of $ZnCl_2$. All three cations, Ni^{2+}, Zn^{2+}, and Ca^{2+}, appear to bind the His-tagged RDDSR FLINT analog, as suggested by the decrease of tryptophan

fluorescence intensity as the concentrations of these cations were increased.

Table III. Fluorescence intensity and anisotropy of His-tagged RDDSR FLINT analog as a function of $NICl_2$, $ZnCl_2$, and $CaCl_2$ [a]

$[NiCl_2]$ uM	Intensity	Anisotropy	[ZnCl2] uM	Intensity	Anisotropy	[CaCl2] uM	Intensity	Anisotropy
0	791325	0.1246	0	881332	0.1446	0	959433	0.1356
5	735267	0.1322	5	879859	0.1326	5	940566	0.1337
10	699807	0.1334	10	847808	0.1369	10	946440	0.1358
20	682581	0.1292	20	821780	0.1523	20	937559	0.1333
40	685818	0.1349	40	813852	0.1860	40	872933	0.1268
80	667912	0.1379	80	780568	0.2137	80	849602	0.1331
100	645837	0.1368	+ 1mM EDTA	739379	0.1269	100	805338	0.1321
200	635790	0.1378				200	778321	0.1369
400	607711	0.1375				400	708589	0.1359

[a] Titration of $ZnCl_2$ was performed in 20 mM Tris, 150 mM NaCl at pH 7.4.

These examples show that divalent cations, such as Ni^{2+}, Zn^{2+}, and Ca^{2+}, interact with FLINT and FLINT analogs. The effect of these cations on the protein appears to be dependent on the nature of the analog. Both Ni^{2+} and Zn^{2+} induce association of FLINT molecules. Zn^{2+} causes

reversible precipitation and can be used in the purification of His-tagged FLINT and analogs.

EXAMPLE 7

IMAC Purification of R218Q from 293 EBNA Cells

FLINT analog R218Q was purified by IMAC chromatography from 3.4 liters of cell culture medium harvested from 293 EBNA cells that expressed R218Q. The FLINT analog was eluted from the IMAC column in a buffer containing PBS, o.5 M NaCl, 0.4 M imidazole, pH 7.4. The eluted material was dialyzed against a buffer containing PBS, 0.5 M NaCl, 10% glycerol, pH 7.4. The dialyzed solution turned cloudy suggesting that the protein had precipitated. This was confirmed experimentally by intrinsic tryptophan fluorescence.

This problem was resolved by resuspending the precipitated protein in PBS, 0.5 M NaCl, 10% glycerol, pH 7.4 and adding EDTA to a final concentration of 50 mM. The precipitated protein went back into solution as observed by visual observation and confirmed by tyrptophan fluorescense.

What is claimed is:

1. A composition comprising a divalent metal cation and a FLINT analog.

2. A composition as in claim 1 wherein said cation is selected from the group consisting of Zn^{+2}, Ca^{+2}, Ni^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, and Cd^{+2}.

3. A composition of Claim 1, wherein the divalent metal cation is Zn^{+2}.

4. A composition of Claim 1, wherein the analog comprises a FLINT glycosylation mutant.

5. A pharmaceutical formulation comprising a composition of claim 1 in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients.

6. A formulation of Claim 5, wherein the total cation concentration is 0.001 to 5.0 mg/mL.

7. A formulation of Claim 6, wherein the total cation concentration is 0.05 to 1.5 mg/mL.

8. A process for reducing aggregation of a FLINT analog molecule comprising the step of removing divalent metal cation.

9. A process as in claim 8 wherein said FLINT analog molecule is in solution.

10.A process as in claim 8 wherein said cation is removed by EDTA.

11. A method for inducing oligomerization of a FLINT analog molecule comprising the step of adding divalent metal cation.

12.A method for inducing aggregation of a FLINT analog molecule comprising the step of adding divalent metal cation until said analog precipitates.

SEQUENCE LISTING

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Tian, Yu
Atkinson, Paul

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/20807

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 C07K1/14 A61K38/17 A61K47/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 30694 A (HUMAN GENOME SCIENCES INC ;FENG PING (US); NI JIAN (US); EBNER REI) 16 July 1998 (1998-07-16) abstract page 42, line 11 - line 22 page 47, line 3 - line 20 sequence of TNFR-6 ---	1-21
A	WO 99 14330 A (GENENTECH INC) 25 March 1999 (1999-03-25) abstract -----	1-21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2000	15/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/20807

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9830694	A	16-07-1998	AU	5815798	A	03-08-1998
			AU	6238698	A	03-08-1998
			BR	9806954	A	21-03-2000
			CN	1247567	T	15-03-2000
			EP	1007659	A	14-06-2000
			EP	0990031	A	05-04-2000
			WO	9830693	A	16-07-1998
WO 9914330	A	25-03-1999	AU	9497098	A	05-04-1999
			EP	1015587	A	05-07-2000